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Phenotype, differentiation, and function differ in rat and mouse neocortical astrocytes cultured under the same conditions

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HIGHLIGHTS

- Primary astrocytes prepared from newborn rats and mice were characterized and compared during 6 weeks in culture.
- We obtained highly enriched rat and murine cultures as most of the cells express the astrocyte markers GFAP and S100β.
- GFAP, nestin and vimentin were expressed at higher mRNA and protein levels in rat than in mouse cultures.
- Rat and mouse astrocytes became differentiated after 6 weeks in culture, but they still expressed nestin at a low level.
- Rat astrocytes kept constant levels of GLAST and GLT-1 over 6 weeks; thus they are suitable for functional studies.

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ABSTRACT

The study of slowly progressing brain diseases in which glial cells play a pathogenic role requires astrocytes that have been cultured for several weeks. We characterized neocortical astrocytes, grown for up to 42 days in vitro (DIV), from newborn rats and mice by indirect immunofluorescence technique, Western blot, and real-time RT-PCR analyses. We obtained highly enriched rat and mouse astrocyte cultures, where most cells were positively stained for the astrocyte markers GFAP, vimentin, and S100β, whereas neuronal and oligodendrocyte markers were undetectable. The protein and mRNA levels of GFAP, vimentin, and nestin were higher in rat than in mouse astrocytes. From 28 to 42 DIV, the levels of vimentin and nestin, but not of GFAP, decreased in both species, with an increase in the vimentin-GFAP ratio of 1.7 for rat, and of 0.9 for mouse astrocytes suggesting that the rat cultures were more differentiated than the mouse cultures, although both remained partially immature. The protoplasmic appearance of the cells, the negative A2B5 immunoreactivity, and the expression of the glutamate transporters GLAST and GLT-1 indicate that the rat and mouse cultures contained mainly type I astrocytes. The protein levels of GLAST and GLT-1 decreased from 28 to 42 DIV in the mouse, but not in the rat astrocytes, suggesting that the rat cultures are suitable for functional studies. Thus, under the same culture conditions, astrocyte cultures from rats and mice differ in phenotype, differentiation, and functionality. This finding should be taken into account when long-lasting glial reaction patterns are being studied.

1. Introduction

Due to their pleiotropic function, astroglial cells in culture are used for many different purposes. For example, they serve as a model to analyze the biological signals regulating their own growth and differentiation (Noble and Murray, 1984; Langan and Slater, 1991), or to investigate the release and formation of astrocyte-released extracellular matrix proteins (Biran et al., 1999). Astroglial cells can also be studied for their physiological importance, e.g. during neuronal differentiation (Schmalenbach and Müller, 1993; Jordan et al., 2008; Costantini et al., 2010; Kremsky et al., 2012), or senescence (Pertusa et al., 2007), as well as for their role in pathophysiological conditions, e.g. in brain tissue grafts (Langan et al., 1995; Krobert et al., 1997), persistent viral infections (Navarra et al.,

Abbreviations: BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindol dihydrochloride; DIV, days in vitro; DMEM, Dulbecco's Modified Eagle's Medium; mGluR, metabotropic glutamate receptor; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline with 0.05% Tween 20.

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2004; Rothenaigner et al., 2007; Porombka et al., 2008), Alzheimer's (Terwel et al., 2011) and Parkinson's diseases (Langan et al., 1995). Finally, astroglial cultures are suitable for studying new therapeutic drugs that either act on astrocytic glutamate receptors (Gallo and Russell, 1995; Cauley et al., 1997) or induce neuroprotective pathways in astrocytes (Ramos et al., 2004). In most of these studies, primary astrocytes had to be maintained in culture for long time periods.

Depending on the requirements of the question being investigated, astrocytes can be obtained from different species, age groups and brain areas. Because the knockout of specific genes via embryonic stem-cell based gene-targeting has already been developed for mice, astrocytes derived from mouse brain are of substantial interest. For rats, on the other hand, the development of this knockout procedure is still in progress (Van Boxtel and Cuppen, 2010). Only a few publications detail the preparation and cultivation of pure astrocytes from mouse brain (Bird, 1983; Eriksson et al., 2000; Sharif et al., 2006). Brown (1998) established a co-culture model where mouse type II astrocytes were maintained for more than 1 year. In some cases, aggregates (Berglund et al., 2004), or organotypic slice cultures (Staal et al., 2011) of the fetal mouse brain are used. However, these culture systems contain astrocytes together with neural precursor cells, oligodendrocytes, and microglia cells and have been shown to lose features of maturity during the culture period. In contrast to cultures from mice, primary astrocyte cultures from rats are well established (McCarthy and de Vellis, 1980; Weinstein, 2001) since the rat brain provides a rich source of astrocyte material. In addition, rat astrocytes are often considered superior when rats are used as in vivo models in the same study. Moreover, long-term astrocyte cultures prepared from rats of different ages (Kremsky et al., 2012), or from different regions such as the cerebral cortex, hippocampus, cerebellum, striatum, optic nerve and spinal cord (Lindsay et al., 1982; Schwartz and Wilson, 1992; Silva et al., 1999; Yang and Hernandez, 2003; Manzano et al., 2007; Brunne et al., 2010), have already been used for comparative studies. The same is true for mice (Liddell et al., 2010).

Prior to the use of astroglial cultures as an in vitro model, the purity and differentiation of the cells during cultivation has to be characterized for each species. When trypsin-digested neocortex is used as a source for the cell culture, neurons, astrocytes (type I and type II), and oligodendrocytes can be identified by specific marker proteins. Maturation of the astrocytes can be analyzed by measuring the levels of A2B5 (present in oligodendroglial precursor cells; Marín-Padilla, 1995), nestin (a marker for immature astrocytes that is also induced upon injury; Bramanti et al., 2010), vimentin, and GFAP (the level of vimentin decreases and that of GFAP increases during differentiation) (Manzano et al., 2007; Bramanti et al., 2010), and the glutamate transporters GLAST and GLT-1, which are present in mature astrocytes (Vermeiren et al., 2005a; Brunne et al., 2010). In this study, astrocyte cultures derived from mouse or rat neocortex were compared for their suitability as a model system on the basis of their differentiation states after 4 and 6 weeks in culture.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium with high glucose and glutamine (DMEM, Cat. 11965), trypsin (Cat. 25050), penicillin–streptomycin (Cat. 15140), the DNase-I kit (Cat. 18068-15), Oligo $(dT)_{12-18}$ Primer (Cat. 18418-012), random primer (Cat. 48190-04), the SuperScript II Reverse Transcriptase Kit (Cat. 18064-022), RNaseOUT (Cat. 10777-019), and the SimplyBlue SafeStain (Cat. LC6060) were purchased from Invitrogen GmbH, Karlsruhe, Germany. Complete Mini-EDTA-free protease inhibitor cocktail (Cat. 04693159001) and Nonidet P-40 (Cat. 11332473001) were from Roche Applied Science, Mannheim, Germany. The reagent 4′,6-diamidino-2-phenylindol dihydrochloride (DAPI, Cat. 6335.1) was obtained from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Triton X-100 (X100), Tween 20 (P1379), dithiothreitol (D0632), bovine serum albumin (BSA, A9418), Kodak BioMax MR films (Z353949-50EA) and the Cell Dissociative Sieve Tissue Grinder Kit, including a screen cup with a retaining ring (S1145), a glass pestle (T8279), a screen replacement key (k3878) and a mesh of stainless steel with an opening size of 73 μm (S4145) were from Sigma–Aldrich, Deisenhofen, Germany. Fetal calf serum (A15-043) and gentamycine (P11-005) were obtained from PAA Laboratories, Marburg, Germany, and the AllPrep RNA/Protein isolation kit was obtained from Qiagen, Hilden, Germany. The IQ™ SYBR Green Supermix (Cat. 170-8884) and Immun-Star™ AP substrate (Cat. 170-5018) were from Bio-Rad (München, Germany). All primary and secondary antibodies used in this study are given in Table 1, including the source, catalogue and lot number, as well as the dilution of the antibodies for the indirect immunofluorescence and the Western blot analysis. Plastic cell-culture flasks (tissue culture treated, Cat. 353136 [75 cm^2], Cat. 353109 [25 cm^2]) and 6-well plates (Cat. 353046) were purchased from BD Falcon, Heidelberg, Germany and Greiner BioOne, Frickenhausen, Germany, respectively. Multitest slides with 6 mm diameter for cell culture and immunofluorescence (Cat. 40-410-06) were from Dunn Laboratories, Asbach, Germany.

2.2. Preparation of rat and mouse cortical astrocytes

Rat and mouse astrocytes were prepared according to McCarthy and de Vellis (1980). Newborn Lewis rats (P1-2, approved by the Government Commission of Animal Care, V54-19C20/15C-GI 18/4) and C57BL/6J mice (P1, Charles River Laboratories, Sulzfeld, Germany) were killed by decapitation, the skull was opened, and the brain was removed. After the meninges were removed from the entire brain, neocortical regions were excised and collected in a Petri dish containing DMEM. The neocortices from both hemispheres of all animals were then transferred into separate tubes (no more than 6 half-cortices per tube) containing 2 ml 0.25% trypsin in DMEM for tissue digestion. After incubation of the tissue for 15 min at 37 °C in a water bath, the trypsin solution was removed, and the tissue was transferred to a metal sieve. Culture medium (DMEM supplemented with 10% fetal calf serum and antibiotics) was then poured over the tissue until all the tissue had passed through the sieve. The collected flow-through from this step was centrifuged at 200 × *g* for 7 min. The resulting cell pellet was re-suspended in culture medium and seeded directly into culture flasks with a ratio of either 6 cortices (from 3 rats) or 16 cortices (from 8 mice) per 75 cm^2. The next day, the astrocytes were washed once with ice-cold phosphate-buffered saline (PBS). The medium was exchanged every 3–4 days.

2.3. Culturing of rat and mouse astrocytes

The cells were passaged the first time shortly before reaching confluence (7–10 DIV) and then again after 3 weeks. Passaging, or splitting, was performed by incubating the monolayer for 8 min with a small amount of 0.25% trypsin (i.e. 1 ml trypsin per 75 cm^2 flask). The detached cells were carefully triturated and directly (no centrifugation step in-between) sub-seeded with a ratio of 1:3 into new flasks. To collect samples of the same batch of cells for either immunocytochemistry, or the parallel isolation of RNA and protein, cells were seeded onto multitest slides and 6-well plates, respectively. This was done after the first passaging for astrocytes of 7 DIV, and after the second passaging for astrocytes of 28 and 42 DIV.